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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>5</sup> :</b> <b>G01N 33/53, C12N 9/66</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 91/18290</b> <b>(43) International Publication Date:</b> 28 November 1991 (28.11.91)
<b>(21) International Application Number:</b> PCT/US91/03252 <b>(22) International Filing Date:</b> 10 May 1991 (10.05.91) <b>(30) Priority data:</b> 522,447 11 May 1990 (11.05.90) US <b>(71) Applicant:</b> GRADUATE HOSPITAL FOUNDATION RESEARCH CORPORATION [US/US]; 415 South 19th Street, Philadelphia, PA 19146 (US). <b>(72) Inventors:</b> KUCICH, Umberto ; 6312 Goyer Street, Philad- elphia, PA 19142 (US). ROSENBLUM, Joel ; 4620 Hazel Avenue, Philadelphia, PA 16143 (US). WEIN- BAUM, George ; 6532 North 12th Street, Philadelphia, PA 19141 (US).		<b>(74) Agents:</b> BERNSTEIN, Alan, H. et al.; Caesar, Rivise, Bernstein, Cohen & Pokotilow, Ltd., 12th Floor, Seven Penn Center, 1635 Market Street, Philadelphia, PA 19103-2212 (US). <b>(81) Designated States:</b> AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH (European patent), CI (OAPI patent), CM (OAPI patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GA (OAPI patent), GB (European patent), GR (European patent), HU, IT (European patent), JP, KP, KR, LK, LU (European patent), MC, MG, ML (OAPI patent), MR (OAPI patent), MW, NL (European patent), NO, PL, RO, SD, SE, SE (European patent), SN (OAPI patent), SU, TD (OAPI patent), TG (OAPI patent).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> IMMUNOASSAY OF ELASTIN PEPTIDES IN URINE  <b>(57) Abstract</b> <p>This invention is a non-intrusive, quantitative urine test of improved sensitivity capable of identifying the presence of lung damage in asymptomatic patients. The invention is useful in monitoring the progression of lung diseases. The method comprises steps of preparing a synthetic peptide or providing elastin-derived peptides and using either the synthetic peptide or the elastin-derived peptides to generate antibodies. The antibodies are then incubated with increasing amounts of the synthetic or elastin-derived peptides in an indirect ELISA. An antibody and peptide mixture is applied to microtiter plates coated with the elastin-derived or synthetic peptide so that the antibodies and peptides combine to form a first complex. The microtiter plates containing the first complex are washed to remove excess unbound antibodies. A known amount of a conjugate of enzyme-antibody is then used and a standardization curve is prepared. An unknown sample of urine with elastin-derived peptides is analyzed in accordance with the above steps. Data are then corrected for creatinine excretion and compared to the standardization curve to determine whether the amount of elastin-derived peptides in the unknown urine sample to establish the existence of lung disease.</p>		

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## IMMUNOASSAY OF ELASTIN PEPTIDES IN URINE

Field of the Invention

This invention relates generally to a method for the immunological identification of elastin-derived peptides (EDP) in urine to assess the existence of lung tissue damage and/or chronic obstructive pulmonary disease (COPD).

Background Art

Chronic obstructive pulmonary disease usually develops over many years, and it is not until lung structure and function have been significantly compromised that COPD can be diagnosed with certainty by radiological and pulmonary function tests.

The connective tissue protein, elastin, is largely responsible for maintaining the elasticity of major blood vessels and lung tissue. In experimental animal models of emphysema, the major emphasis in research has been the study of the destruction of mature elastin fibers by administration of selective proteases by aerosol or intratracheal instillation. In these systems, there is a strong correlation between the production of emphysema and the digestion of insoluble amorphous lung elastin. Similarly, destruction of elastin fibers in humans is a likely prerequisite for the development of COPD and/or emphysema.

An early study involving the pathogenesis of destructive lung disease involved the immunologic identification of peptides derived from lung elastin appearing in blood or urine. See Harel, S., Janoff, A., Yu, S.U., Hurewitz, A., Bergofsky, E.H., Measurement of Elastin in Degradation in vivo by Desmosine Radioimmunoassay, Am.Rev.Respir.Dis. 122:769-73 (1980); which describes the use of a radioimmunoassay to quantify the characteristic elastin cross-link, desmosine, in human urine and found significantly higher levels in emphysematous patients compared to normal controls. However, more recent studies failed to detect any difference in urine desmosine content between individuals with normal lung function and those with obstructive lung disease. See Davies, S.F., Offord, K.P., Brown, M.G., Campe, H.,

Niewoehner, D., Am.Rev.Respir.Dis. 128:473-75 (1983), which found that urine desmosine is unrelated to cigarette smoking or to spirometric measurements of lung function.

In Kucich, U., Christner, P., Weinbaum, G., Rosenbloom, J. (hereinafter Kucich, I), Immunologic Identification of Elastin-Derived Peptides in the Serums of Dogs with Experimental Emphysema, Am.Rev.Respir.Dis. 122:461-65 (1980), the present inventors and others used antibodies against elastin-derived peptides (EDP) to detect elastin peptides in the sera of animals with experimental emphysema. See also Darnule, T.V., Osman, M., Darnule, A.T., Mandl, I., Turino, G.M., Immunologic Detection of Lung Elastin Peptides in the Serum of Rats with Elastin Induced Emphysema, Am.Rev.Respir.Dis. 121:331 (1980).

In an analysis of human plasma by an enzyme-linked immunosorbent assay (ELISA), the three present inventors with others demonstrated that significantly higher values of elastin peptides were found in emphysema patients than in individuals with normal lung function. Kucich, U., Christner, P., Lippmann, M., Kimbel, P., Williams, G., Rosenbloom, J., Weinbaum G., Utilization of a Peroxidase-Antiperoxidase Complex in an Enzyme-Linked Immunosorbent Assay of Elastin-Derived Peptides in Human Plasma, Am.Rev.Respir.Dis. 131:709-13 (1985), (hereinafter Kucich II). See also Am.Rev.Respir.Dis. 127:S28-30 (1983).

The analysis of human sera by radioimmunoassay has previously demonstrated that significantly higher values of elastin peptides were found in emphysema patients than in individuals with normal lung functions. Darnule, T.V., McKee, M., Darnule, A.T., Turin, G.M., Mandl, I., Anal.Biochem. 122:302-07 (1982).

While the Kucich II and the Darnule studies suggest the possible utility of such tests, their value in monitoring the pathogenesis of destructive lung disease remains to be proven. In the case of Kucich II, the patients with COPD had elevated peptide levels ( $127 \pm 47$  ng/ml) as compared to normal non-smokers ( $58 \pm 17$  ng/ml), while normal smokers had intermediate values (76

$\pm 42$  ng/ml). With a small sample number, PiZZ individuals also appeared to have elevated levels ( $93 \pm 18$  ng/ml). These peptide levels however, lack sensitivity, particularly with the large variation of values in patients with COPD.

While it has been demonstrated that the monospecific antibody used in the plasma assay performs satisfactorily, it is believed that the use of a monospecific antibody in a urine assay has yet to be successfully employed. In prior work performed by the present inventors in developing a plasma test for detecting and monitoring COPD, they successfully demonstrated a significant elevation in antigenic reactivity using a monospecific antibody in samples from subjects with COPD (average of 432 ng/ml equivalents) compared to controls (108 ng/ml equivalents). In contrast however, the present inventors have not been able to successfully develop the parallel use of a monospecific antibody in a comparable urine test for COPD and lung damage testing.

For an extended time period, many researchers believed that a similar immunological test for urine was impractical, if not impossible, as reinforced by the failure of the urine desmosine studies noted herein. This theory was held, in part, because scientists believed that the peptides degraded from elastin and circulating in the plasma, would be further degraded by kidney filtration and other bodily processes, so that they would be effectively undetectable in urine, thereby preventing the development of a reliable quantitative test.

There has therefore been a long-felt need for a urine test which can quickly, easily and inexpensively determine the presence of lung tissue damage, emphysema, and/or COPD. Using urine as a test fluid is less intrusive to the patient and permits a larger sample to be collected and used, and is therefore preferable to using plasma and other more complex methods.

#### Objects of the Invention

Accordingly, it is a general object of the instant invention to provide a non-intrusive, quantitative test of improved sensitivity that is capable of identifying the presence

of lung damage at an early stage, in asymptomatic individuals. This test would be useful in identifying individuals who are at risk of developing emphysema and COPD, and in monitoring disease progression.

#### Summary of the Invention

These and other objects of this invention are achieved by providing a non-intrusive, quantitative urine test of improved sensitivity that is capable of identifying the presence of lung damage at an early stage, before symptoms develop. Such a quantitative test of improved sensitivity is useful in identifying individuals who are at risk of developing emphysema. Such a test may also be used in monitoring the progression of the disease. The method comprises the steps of preparing a synthetic peptide or providing elastin-derived peptides and using either the synthetic peptide or the elastin-derived peptides to generate antibodies. The antibodies are then incubated with increasing amounts of either a synthetic peptide or the elastase-digested elastin-derived peptides in an indirect ELISA.

A mixture of antibodies and peptides is then applied to microtiter plates coated with the elastase-digested elastin-derived peptides or synthetic peptides so that the antibodies and peptides combine to form a first complex. The microtiter plates containing the first complex are washed to remove excess unbound antibodies. A known amount of a conjugate of enzyme-antibody that is very specific and will cling only to bound antibodies is then used. A standardization curve based on the foregoing steps is then prepared.

A sample of urine containing an unknown amount of elastin-derived peptides is obtained and analyzed in accordance with the above steps. The results of such analysis are corrected for creatinine excretion and compared to the standardization curve. From these results one can determine whether the amount of elastin-derived peptides in the unknown urine sample is sufficiently in excess of a standard value in the urine of a group of control individuals not having lung damage or chronic

obstructive pulmonary disease, to establish the existence of lung disease or chronic obstructive pulmonary disease.

The kit of the above invention for carrying out a method for immunologic detection of elastin-derived peptides in urine comprises a synthetic peptide or natural peptide, and is employable with elastin-derived peptides prepared from the amorphous component of human lung elastin. The kit includes a method to use the synthetic peptide in an indirect ELISA to quantify the elastin-derived peptides in a urine sample, and to compare the results obtained with an established standard.

#### Description of the Drawings

Fig. 1 is a plot of the molecular sieving of a urine sample which illustrates the elution of elastase-derived elastin-derived peptides in a solution containing known molecular weight standards.

Fig. 2 is a bar graph showing the quantity of elastin-derived peptides determined in a group of individuals comprising a non-smoker control group, a smoker group and a group of COPD patients, in accordance with the method of the instant invention.

#### Detailed Description of the Preferred Embodiment

##### Materials and Methods

Goat anti-rabbit (GAR) serum may be obtained from Cappel Laboratories of Cochranville, PA. Rabbit peroxidase-antiperoxidase (PAP) complex may be obtained from Sternberger Meyer Immunocytochemicals, Inc. of Jarrettsville, MD. Immulon #2 Microtiter plates may be obtained from Scientific Accessories of Andalusia, PA. Other chemicals should be of reagent grade.

##### I. Preparing the Synthetic Peptide for Antibody Production

To generate antibodies for use in the present invention, it is necessary to prepare either a synthetic peptide (perhaps with the amino acid sequence GFPGGACLGKACGRKRK) or elastin-derived peptides from human lung elastin (to be described below). The following table sets forth the amino acid which

corresponds to each one-letter symbol in the preceding amino acid sequence:

<u>One-Letter Symbol</u>	<u>Amino Acid</u>
A	Alanine
C	Cysteine
F	Phenylalanine
G	Glycine
K	Lysine
L	Leucine
P	Proline
R	Arginine
S	Serine

To prepare the antibodies against the synthetic peptides, a synthetic amino acid sequence, such as GFPGGACLGKACG-RK RK may be coupled to keyhole limpet hemocyanin (KLH) using glutaraldehyde. This complex may then be used to produce antibodies for use in the method of the present invention as described below. Baron, M.H., and Baltimore, D., Antibodies Against a Chemically Synthesized Genome-Linked Protein of Polio Virus React With Native Virus-Specified Proteins, Cell, (1982).

II. In Vitro Preparation of Elastin-Derived Peptides Using Elastase for the Production of Antibodies

To prepare the elastin-derived peptides (EDP) for the production of antibodies as described below and as previously described in Kucich I, the amorphous component of human lung elastin may be digested with human neutrophil elastase at a 1:500 ratio of enzyme to elastin (w/w) for 24 hours at 37°C.

III. Preparing Elastin Peptides from Urine in Vitro for the Production of Antibodies

In accordance with established practices and methods for producing antibodies, it is believed that the antibodies to elastin-derived peptides isolated from urine may be prepared by the following procedure, in accordance with the preferred method of the present invention. Preparation and use of antibodies against the antigens isolated from urine should result in increased sensitivity of the present invention, as these anti-



bodies are believed to be more specific to the EDP antigen in an unknown sample of urine.

A 50-100 ml sample of human urine containing elastin-derived peptides can be concentrated 10-fold by ultrafiltration in an Amicon cell Model 52, and should result in an approximate recovery of 60-70% of the elastin-derived peptides present in the urine sample. The urine sample can then be centrifuged in a Sorvall refrigerated centrifuge, DuPont Company, RC-5B rotor, at 12,000 rpm for 10 minutes to remove solids. The supernatant should then be removed and frozen at -20°C as the elastin-derived peptides may be unstable at 4°C. The urine sample will then be fractionated on a molecular sieve P-10 column, as shown in Fig. 1, and should result in a more than 90% recovery of elastin derived peptides.

Fig. 1 demonstrates the fractionation of a human urine sample by molecular sieving on the P-10 column, using an eluting solution of 0.2 M phosphate buffer, 0.2% sodium azide, pH = 7.2, along with the conventional known molecular weight standards of bovine serum albumin (BSA), soy trypsin inhibitor (SBTI) and cytochrome C (CYTO C). Fractions of 5 mls were collected and absorbance was read at 280 nm to provide EDP concentrations. It is believed that ninety percent of the elastin immunoreactivity should elute between 4-20,000 daltons with little contaminating protein.

The desired fractions can then be collected and the sample may then be placed on a Superose 12 liquid chromatography column (FPLC) which separates materials according to molecular weight. It is expected that desired column effluent fractions will contain peptides having a molecular weight in the range of 13,000 daltons. Peptides having a molecular weight in this range exhibit the desired activity when subjected to peptide detection analysis. These purified EDP from urine can then be used to generate antibodies in accordance with the method of Section IV below.

#### IV. Generating Antibodies to the Synthetic or Elastin-Derived Peptides

After preparing the synthetic or elastin-derived peptides as described above, either type may be used to generate antibodies in New Zealand white rabbits. See Rosenbloom, J., Kucich, C., Weinbaum, G., Kimbel, P., and Feierstein, N., *Immunologic Identification of Carboxy Terminal Sequences of Elastin in Human Plasma Using Monospecific Antibodies, Pulmonary Emphysema and Proteolysis*, Vol. 2, edited by Taylor, J.C., and Mittman, C., Academic Press (1986). The IgG fractions may be purified by ammonium sulfate precipitation followed by DEAE chromatography.

#### V. The Enzyme-Linked Immunosorbent Assay (ELISA)

Microtiter plates may be coated with 200  $\mu$ l per well of elastin-derived peptides (250 ng/ml) or synthetic peptide (1000 ng/ml), by incubation at 4°C for 24 hours in 0.1 M carbonate, pH = 9.6, containing 0.02% M sodium carbonate. Standard curves for the indirect ELISA may be generated by incubating 5  $\mu$ g/ml primary antibody (for the elastin-derived peptides) or 7  $\mu$ g/ml primary antibody (for the synthetic peptide) with variable concentrations of competing antigen for 16 hours at 4°C. These reaction mixtures (200  $\mu$ l per well) may be transferred to the coated wells and incubated at 4°C for 1 hour. The wells may be washed with phosphate buffered saline (PBS-Tween 20), and afterwards 200  $\mu$ l per well of goat antirabbit serum at a dilution of 1:2000 may be added to the wells and incubated for 1 hour at room temperature. After washing, 200  $\mu$ l per well of the peroxidase-antiperoxidase complex may be added to the wells at a dilution of 1:2000 in PBS-Tween 20, and incubated for 30 minutes at room temperature. The wells may be washed with PBS-Tween 20 and then 200  $\mu$ l per well of a 2 mg/ml solution of o-phenylenediamine and 0.006% H<sub>2</sub>O<sub>2</sub> in 0.1 M citrate, pH = 4.5, may be added. After 45-60 minutes, the absorbance values may be determined at 450 nm using an automatic plate reader (Flow Laboratories, McLean, VA). Unknown urine samples may preferably be analyzed in triplicate at two different concentrations (usually at 30 mls 1:20 and 60 mls 1:10 dilu-

tions). See also the procedure and materials of the ELISA of Kucich II.

#### VI. Preparing Urine for Analysis

A 50-100  $\mu$ l sample of human urine containing an unknown amount of elastin-derived peptides is obtained after centrifugation of the collected urine sample in a Sorvall refrigerated centrifuge, DuPont Company, RC-5B sized rotor, at 12,000 rpm for 10 minutes to remove solids. The supernatant may then be removed and frozen at  $-20^{\circ}\text{C}$ , as the elastin-derived peptides may be unstable at  $4^{\circ}\text{C}$ . The urine is then concentrated 10-fold by ultrafiltration in an Amicon cell Model 52, and it is believed that there will be a 60-70% recovering of elastin-derived peptides.

The human elastin-derived peptides or the synthetic peptides may be applied to microtiter plates. The EDP antibodies and peptides (synthetic or urine-derived) should combine to form a complex. The microtiter plates may then be washed to remove any excess unbound antibodies. After binding the enzyme-linked antibodies and the addition of substrate, the absorbance of each unknown is then subjected to the standardization curve previously prepared in order to determine the elastin peptide level in the urine sample. By comparing the concentration of elastin-derived peptides in the urine sample, after correction for creatinine excretion, to those readings obtained from a normal individual, one can establish the existence of chronic obstructive pulmonary disorder, as shown more clearly in Fig. 2.

The results of the above analysis should demonstrate that COPD patients excrete high concentrations of low molecular weight EDP in their urine. In the study of a group of individuals comprising non-smokers, smokers and COPD patients, in accordance with the method of the above invention, the following results were obtained as summarized in Fig. 2 ( $n$  = number of individuals) and in Table 1 below (values reported below as ngEDP/mg creatinine):

Table 1

<u>Non-Smokers</u>	<u>Smokers</u>	<u>COPD</u>
62	110	211
9	125	20
94	7	441
58	139	255
36	55	275
106	37	329
66	53	296
30	171	123
24		84
142		364
17		350
108		515
178		165
180		222
24		126
36		202
200		324
102		77
61		181
42		17
91		84
120		520
123		
209		

Non-smokers n=24

Smokers n=8

COPD n=22

After correction for creatinine excretion, the present inventors determined that EDP levels for COPD patients were  $252 \pm 162$  ng/mg creatinine (n=22), for non-smokers EDP levels were  $88 \pm 60$  ng/mg (n=24;  $p < 0.001$ ) and for normal smokers  $87 \pm 57$  ng/mg (n=8). Multiple samples from the same patient revealed that urinary EDP levels varied less than 20-25%. COPD patients appeared to excrete lower molecular weight peptides into the urine, since dialysis through a 3500 M.W. cut-off membrane resulted in the loss of 80% of the immunoreactivity, while the urine of a non-smoker lost less than 35% immunoreactivity. Little EDP antigen may be detected in any urine using an elastin carboxy-terminus antibody, which detects EDP in plasmas of non-

smokers, smokers and COPD individuals. Am.Rev.Resp.Dis. 137:A372 (1988). These data suggest considerable antigen processing of EDP prior to urinary excretion, particularly in COPD patients. The easy sampling and the presence of low molecular weight EDP make the urine ideal to use for isolation and characterization of such elastin antigen(s). Identifying the in vivo - generated elastin antigen(s) may provide tools for developing more sensitive probes of lung destruction and for monitoring therapy.

Without further elaboration the foregoing will so fully illustrate my invention that others may, by applying current or future knowledge, readily adapt the same for use under various conditions of service.

CLAIMS

We claim:

1. A method of determining the presence of elastin derived peptides in urine to identify the occurrence of actual or potential lung damage in asymptomatic humans and to detect and monitor chronic obstructive pulmonary disease, said method comprising the steps of:

- (a) preparing a synthetic peptide or providing one or more elastin-derived peptides;
- (b) using either said synthetic peptide or one or more of said elastin-derived peptides to generate antibodies;
- (c) using said antibodies, including incubating said antibodies with increasing amounts of either said synthetic peptide or one or more of said elastin-derived peptides;
- (d) applying a mixture of said antibodies and one or more of said peptides to microtiter plates coated with said elastin-derived peptides or coated with said synthetic peptide whereby said antibodies and peptide(s) combine to form a first complex;
- (e) washing said microtiter plates containing said first complex to remove excess unbound antibodies;
- (f) using known amounts of a conjugate of enzyme-antibody that is very specific and will cling only to said bound antibodies;
- (g) preparing a standardization curve based on the foregoing steps;
- (h) obtaining a sample of urine containing an unknown amount of elastin-derived peptides;
- (i) subjecting said sample of urine to steps (c), (d), (e), (f) and (g) hereinabove,

- (j) correcting the results from step (i) for creatinine excretion; and
- (k) determining whether the amount of elastin-derived peptides in said sample is sufficiently in excess of a standard value in the urine of an individual not having lung damage or chronic obstructive pulmonary disease, to establish the existence of lung disease or chronic obstructive pulmonary disease.

2. The method of Claim 1 characterized in that the urine sample is taken from a person suspected of being afflicted with chronic obstructive pulmonary disease (COPD).

3. A kit for carrying out a method for immunologic detection of elastin-derived peptides in urine, said kit comprising a synthetic peptide or natural elastin-derived peptide, said kit being employable with elastin-derived peptides prepared from the amorphous component of human lung elastin, said kit including means to use said synthetic peptide or said natural elastin-derived peptide in an indirect ELISA to quantify the elastin-derived peptides in a urine sample, and means to compare the results obtained with an established standard.

FRACTIONATION OF HUMAN URINE BY MOLECULAR SIEVING

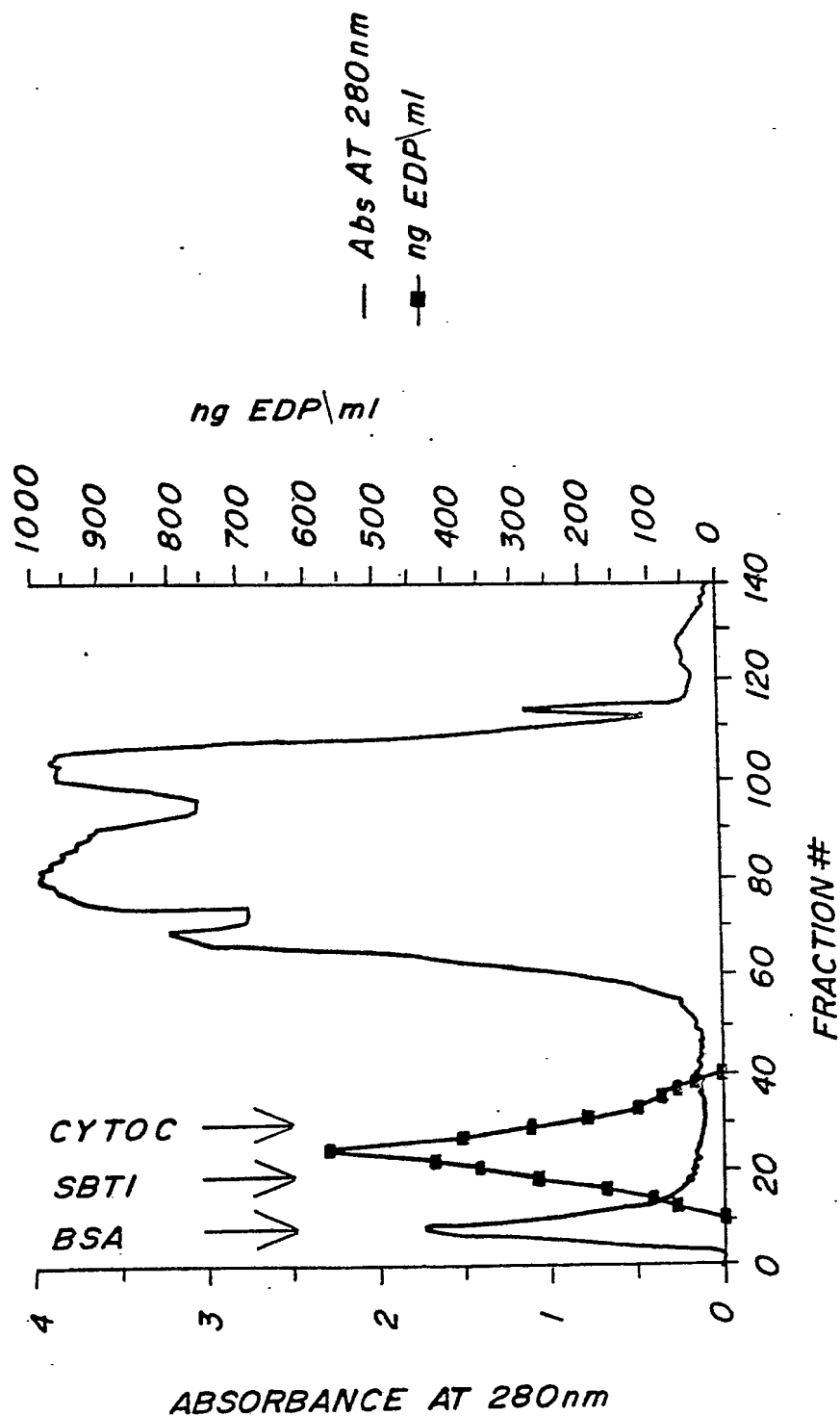


FIG. 1



ELASTIN PEPTIDE DISTRIBUTION IN HUMAN URINE

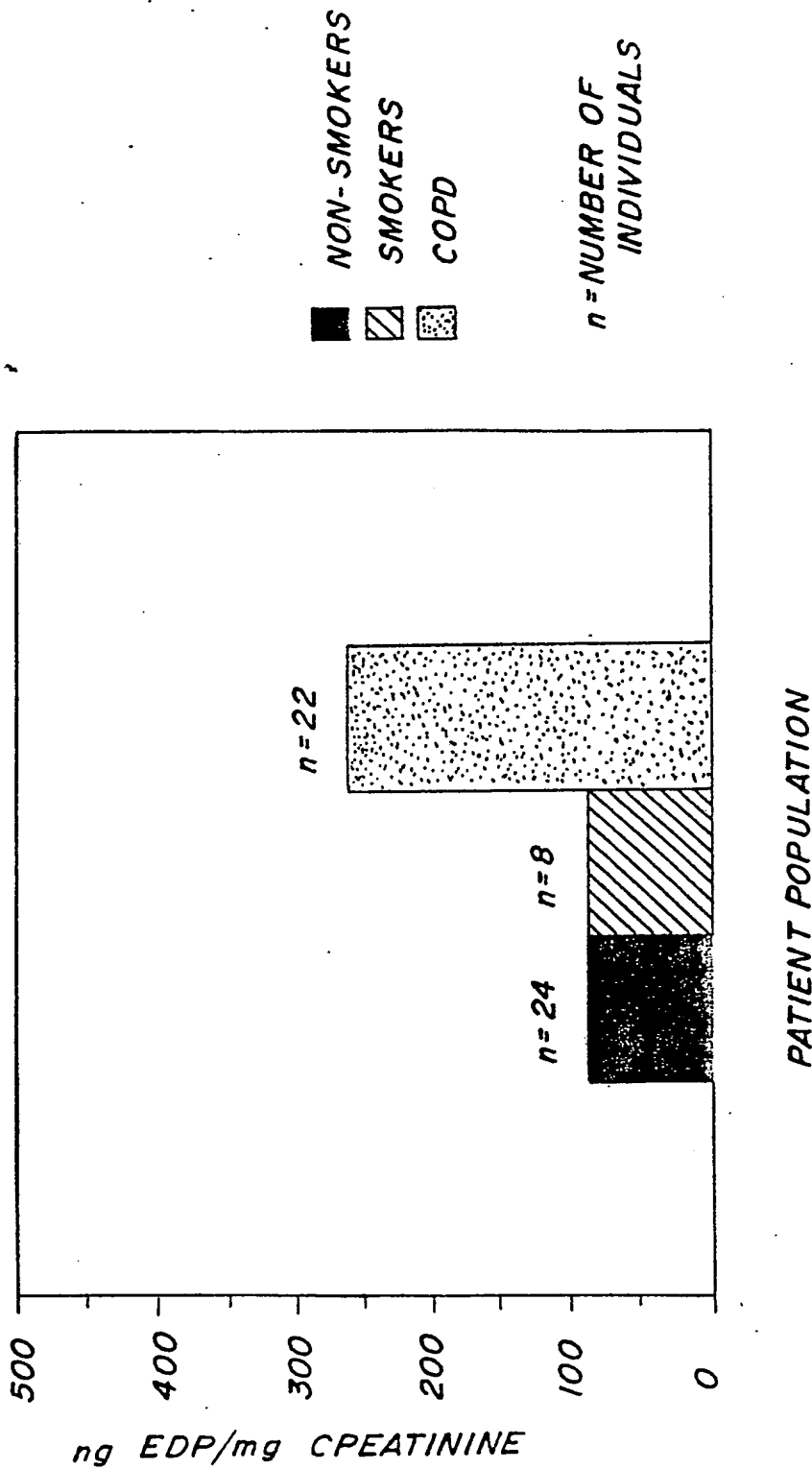


FIG. 2

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/03252

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all <sup>6</sup> )		
According to International Patent Classification (IPC) or to both National Classification and IPC		
U.S.Cl.: 435/7.93; 435/218		
IPC (5): G01N 33/53; C12N 9/66		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
U.S.Cl.	435/7.93; 435/218	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT <sup>9</sup></b>		
Category <sup>1</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X	JOURNAL OF IMMUNOLOGICAL METHODS, Vol. 107, No. 1, issued 1988, P. Laurent et al., "Quantitation of elastin in human urine and rat pleural meothelial cell matrix by a sensitive avidinbiotin ELISA for desmosine, "pages 1-11, see entire article.	1-3
Y	JOURNAL OF CLINICAL INVESTIGATION, Volume 61, 1978, R.A. Goldstein et al., "Urinary ecretion of elastin peptides containing desmosine after intratracheal injection of elastase in hamsters, "pages 1286-1290, see figure 2.	1-3
Y	BIOCHEMISTRY, Volume 25, 1986, D.S. Wrenn et al., "Characterization of biologically active domains on elastin: identification of a monoclonal antibody to a cell recognition site," pages 5172-5176, see entire article.	1-3
Y	CHEMICAL ABSTRACTS, Volume 106, No. 19, issued 11 May 1987, J. Rosenbloom et al., "Newly determined carboxy terminal sequences in tropoelastin: immunologic identification insoluble elastin, "see abstract 151824, Collagen Related Research, 6(5), 423-33.	1-3
<p>* Special categories of cited documents: <sup>10</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
04 September 1991	12 SEP 1991	
International Searching Authority	Signature of Authorized Officer	
ISA/US	Michael Woodward	

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## FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	CHEMICAL ABSTRACTS, Volume 107, No. 21, issued 23 November 1987, J. Rosenbloom et al., "Immunologic identification of carboxy terminal sequences of elastin in human plasma using monospecific antibodies," see abstract 194478, Pulm. Emphysema Proteolysis, 1986, (Conf.) 1986, 245-54.	1-3
X	CHEST, Volume 96, No. 2, Supplement, issued August 1989, E.E. Schriver et al., "Elastin fragment levels in human plasma, urine, and bronchoalveolar lavage fluid (BALF)," page 153S, see abstract.	1-3
X,P	U. Kucich et al., "Urine from emphysema patients contains elevated levels of elastin-derived	1-3

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE<sup>1</sup>

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers \_\_\_\_\_, because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claim numbers \_\_\_\_\_, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out<sup>2</sup>, specifically:
3. ☐ Claim numbers \_\_\_\_\_, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING<sup>2</sup>

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

## Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	petides," 1990 World Conference on Lung Health, see abstract, AM. REV. RESPIR. DIS. 141 (4 Part 2), A232.	
Y	AMERICAN REVIEW RESPIRATORY DISEASE, Volume 131, issued 1985, U. Kucich et al., "Utilization of a peroxidase antiperoxidase complex in an enzyme-linked immunosorbent assay of elastin-derived peptides in human plasma," pages 709-713, see entire document.	1-3

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